Cooperative Interactions Between RB and p53 Regulate Cell Proliferation, Cell Senescence, and Apoptosis in Human Vascular Smooth Muscle Cells From Atherosclerotic Plaques

Martin R. Bennett, Kirsty Macdonald, Shiu-Wan Chan, Joseph J. Boyle, Peter L. Weissberg

Abstract—Compared with vascular smooth muscle cells (VSMCs) from normal vessels, VSMCs from human atherosclerotic plaques proliferate more slowly, undergo earlier senescence, and demonstrate higher levels of apoptosis in culture. The tumor suppressor genes $p105^{RB}$ (retinoblastoma, acting through the E2F transcription factor family) and p53 regulate cell proliferation, cell senescence, and apoptosis in many cell types. We have therefore determined whether these stable growth properties of plaque VSMCs reflect altered activity of RB and/or p53. VSMCs were derived from coronary atherectomies or from normal coronary arteries from transplant recipients. Compared with normal VSMCs, plaque VSMCs showed a higher ratio of the active (hypophosphorylated) to the inactive (phosphorylated) form of RB and a lower level of E2F transcriptional activity. Cells were stably transfected with retrovirus constructs that inhibited RB or p53 alone or in combination. Suppression of RB alone increased rates of cell proliferation and apoptosis and inhibited cell senescence in normal VSMCs. Suppression of p53 and RB together had similar effects but, additionally, resulted in immortalization of normal VSMC cultures. In contrast, inhibition of RB binding to E2F or ectopic expression of E2F-1 in plaque VSMCs induced massive apoptosis, which required suppression of p53 to rescue cells. Suppression of RB and p53 together increased cell proliferation and delayed senescence but failed to immortalize plaque VSMCs. Inhibition of p53 alone had minimal effects on plaque VSMCs but increased the lifespan of normal VSMCs. We conclude that human plaque VSMCs have slower rates of cell proliferation and earlier senescence than do cells from normal vessels because of a defect in phosphorylation of RB. Furthermore, both disruption of RB/E2F and inhibition of p53 are required for plaque VSMCs to proliferate without apoptosis. This observation may explain the relatively low level of cell proliferation and high level of apoptosis seen in VSMCs in human atherosclerotic plaques. **(*Circ Res.* 1998;82:704-712.)**

Key Words: atherosclerosis ■ apoptosis ■ retinoblastoma ■ p53

Atherosclerosis is characterized by an intimal accumulation of VSMCs, together with macrophages and T lymphocytes, and both intracellular and extracellular lipid. Excess VSMC proliferation has been proposed in the pathogenesis of atherosclerosis, although labeling indices for cell proliferation in atherosclerotic plaques are low.[1,2] Furthermore, VSMCs removed from human plaques demonstrate no excess proliferation in culture compared with cells from normal vessels. In fact, VSMCs derived from human atherosclerotic plaques show three characteristic properties in culture. First, VSMCs proliferate slowly, with a lower percentage of cells in S phase at any time compared with VSMCs from normal vessels.[3–5] Second, plaque VSMC cultures undergo senescence, defined by no detectable proliferation over a prolonged period (up to 1 week) at two to five passages, five to eight passages earlier than do cells from normal vessels.[4,5] Finally, plaque VSMCs show higher levels of spontaneous apoptosis than do normal VSMCs.[5] Indeed, the recent identification of apoptotic VSMCs in human plaques[6,7] has emphasized that VSMC number in the atherosclerotic plaque is regulated by both cell proliferation and apoptosis.

A number of gene products are upregulated when mesenchymal cells senesce and are implicated in mediating the terminal G_0/G_1 arrest. These include the tumor suppressor genes $p105^{RB}$ and $p53$[8–11] and the p53 target genes p21 and GADD45.[12–16] RB exerts most of its growth arrest action by forming complexes with members of the E2F transcription factor family,[17,18] resulting in inhibition of E2F transactivation of genes necessary for S phase.[19–24] Phosphorylation of RB in late G_1 by cdk/cyclin complexes releases E2F,[20] allowing S-phase entry. In contrast, overexpression of p53 results in G_1 or G_2/M arrest mediated by transcriptional activation of target genes such as p21, an inhibitor of cdks,[25] or GADD45, which suppresses critical enzymes in DNA synthesis.

RB, p53, p21, and GADD45 regulate cell cycle progression in actively proliferating cells,[14,26] and both RB and p53 also

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From the Department of Medicine, Addenbrooke's Hospital, Cambridge, UK.
Correspondence to Dr Martin R. Bennett, Unit of Cardiovascular Medicine, University of Cambridge Clinical School of Medicine, Department of Medicine, Level 5 Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QQ, UK.
E-mail mrb@mole.bio.cam.ac.uk

Selected Abbreviations and Acronyms

CAT = chloramphenicol acetyltransferase
cdk = cyclin-dependent kinase
DN-p53 = dominant-negative p53 minigene
HPV = human papilloma virus
RB = retinoblastoma tumor suppressor gene
SV = simian virus
VSMC = vascular smooth muscle cell

regulate apoptosis.[27–30] Indeed, apoptosis due to E2F overexpression is dependent on p53.[31–34] We therefore postulated that perturbed activity of RB and p53 are responsible for the slower proliferation, earlier senescence, and higher rates of apoptosis in plaque VSMCs than in VSMCs from normal vessels. In accordance with this hypothesis, we have previously demonstrated that plaque VSMCs are more sensitive to p53-mediated apoptosis than are normal VSMCs.[35] However, p53 expression and activity were similar in plaque and normal VSMCs, and inhibition of p53 alone did not increase the proliferation of plaque VSMCs.[35] Therefore, the purpose of the present study was to examine the role of RB in regulating plaque VSMC proliferation and apoptosis.

Materials and Methods

Cell Isolation and Culture

Plaque cells were derived from primary coronary artery plaques by atherectomy, and normal medial VSMCs were obtained from coronary arteries of patients undergoing cardiac transplantation for nonischemic cardiomyopathy as part of the University of Washington Heart Transplantation Program. The isolation and characterization of these cells has already been described.[5] Briefly, cultures were obtained from specimens from individual patients of both sexes with a range of ages between 34 and 62 years (mean age of patients was 49.8 years [plaque specimens] and 47.8 years [normal coronary artery specimens]). Plaque cultures were obtained from patients with either stable or unstable angina, since we have previously demonstrated that cells from both types of lesions undergo similar rates of apoptosis in culture.[5] Characterization of VSMCs as intimal (from atherectomy tissues) and medial (from normal vessels) was performed by hematoxylin and eosin histology of the atherectomies or arteries removed (not shown). Cells were characterized as VSMCs by culture morphology and immunocytochemical staining pattern at passage 3 (α-actin positive, vimentin positive, von Willebrand factor negative, desmin negative, and smooth muscle myosin positive) (not shown). Three independent cultures of plaque-derived or normal smooth muscle cells from three different patients were used for gene transfer. Cells from atherectomy specimens and from normal vessels were cultured in medium 199 containing 10% FCS and 10 mmol/L HEPES (Sigma Chemical Co) and equilibrated with 95% air/5% CO_2. Cultures from individual patients were maintained separately in medium containing 10% FCS for the whole culture period. Cells were passaged by trypsinization in 0.05% trypsin in PBS at confluence, with a split ratio of 1:3 at all times.

Inhibition of RB or p53 Activity in Human VSMCs

To examine the effects of RB or p53 in VSMCs, we generated cell lines in which RB or p53 activity was inhibited by virus genes HPV type 16 E6 or E7 or SV40 large T antigen, in addition to the minigene DN-p53. This minigene encodes a protein that contains only the C-terminus of the protein (amino acids 302 to 370). The resulting protein can therefore dimerize with wild-type p53 protein, but the dimer cannot bind DNA. This results in a suppression of p53-mediated transcriptional activity.[35,36] Full-length cDNAs encoding SV40, HPV E6, HPV E7, HPV E6/E7, E2F-1, or DN-p53 were cloned into the retrovirus vector pBabe (neo/puro). Ecotropic retroviruses containing these genes or the retrovirus vector alone were generated by calcium phosphate transfection of the vector containing the gene of interest into an ecotropic packaging cell line, GP+E86.[37] The medium was then harvested from these cells after 48 hours and used to infect a second generation packaging cell line, PA317, in medium containing hexadimethrine bromide (8 μg/mL). Packaging cells were selected in 500 μg/mL of G418 (Geneticin, GIBCO) or 2.5 μg/mL of puromycin (Sigma), until a confluent 10-cm-diameter dish of resistant cells was obtained. Amphotropic virus was then harvested from the medium of this dish after 48 hours of incubation. This concentrated retrovirus stock was then used to infect human VSMCs as previously described.[5] Resistant human VSMCs were selected in 750 μg/mL of G418 or 2.5 μg/mL of puromycin after at least 4 weeks in antibiotic-containing medium. Antibiotic-resistant cells from each individual patient after retrovirus infection were pooled and used for experiments rather than clones of antibiotic-resistant cells. This approach avoids clonal variation in gene expression and also is necessary in view of the difficulty in cloning human VSMCs.

Time-Lapse Videomicroscopy

Cells were prepared for videomicroscopy as previously described.[5] Briefly, cells were maintained in medium containing 10% FCS, washed three times in medium containing 0% FCS, and then cultured in this latter medium. Flasks were gassed with 95% air/5% CO_2 every 24 hours and sealed. An Olympus OM-70 microscope was enclosed in a plastic environment chamber and maintained at 37°C by an external heater. The time-lapse equipment consisted of a Sony 92D CCD camera with a Panasonic 6730 time-lapse video recorder. Films were analyzed for morphology of apoptosis and cell death rates as previously described[5] using an observer blind to cell type and treatment conditions. Apoptotic cell death events were scored midway between the last appearance of normality and the point at which the cell became fully detached and fragmented, an interval of typically 60 to 90 minutes. Cell division was scored at the time at which septa appeared between two daughter cells. Each individual cell culture was analyzed in duplicate as a minimum (plaque VSMCs, n=8; normal coronary VSMCs, n=8). Time-lapse videomicroscopy was also used to establish culture senescence, defined as no detectable cell proliferation over 7 days, together with no detectable S-phase percentage on flow cytometry (see below).

Western Blotting for RB, p107, and p130

Western blots were prepared by lysis of cells cultured in medium containing 10% FCS. Protein isolation, electrophoresis, and blotting were as previously described.[38] RB was detected using a mouse anti-human RB monoclonal antibody that recognizes both phosphorylated and hypophosphorylated forms of RB (No. 14001A, Pharmingen), although the phosphorylated form runs at a higher point on the gel. We examined expression of the hypophosphorylated form of RB alone by using an antibody that recognizes only this form (No. 14441A, Pharmingen), and we also examined expression and phosphorylation status of the RB family members p130 and p107 (No. sc-317, Santa Cruz, and No. 14911A, Pharmingen, respectively) according to the manufacturers' instructions. Protein concentrations were assessed by modified Bradford assay (Bio-Rad) before loading.

Assay of E2F or p53 Transcriptional Activity

To assess E2F or p53 activity in native and infected cell lines, uninfected VSMCs (at 75% confluence in a 10-cm dish) from each patient or the cell lines were transfected using 10 μg of the E2F reporter plasmid E2F-CAT or the p53 reporter construct p53-CAT by calcium phosphate transfection and glycerol shock.[37] The reporter constructs were constructed using three copies of the E2F binding sequence TTTCGCGC from the human cdc2 promoter upstream from CAT. The p53-CAT construct uses three repeats of the p53 consensus binding sequence TGCCT linked to the CAT gene.[39] After 48 hours, cells were harvested and lysed, and CAT activity was

TABLE 1. Baseline Characteristics of Cell Proliferation, Apoptosis, and Cell Senescence of Plaque and Normal VSMCs

	No. of Divisions/24 h	No. of Deaths/24 h	S Phase, %	Passage Number at Senescence
Normal VSMCs	14.9±2.2	0.2±0.05	8.5±1.1	8–10
Plaque VSMCs	4.2±1.2	3.2±1.0	3.5±1.2	3–6

Values are mean±SD (n=3).

determined using standard assay conditions with [^{14}C]chloramphenicol.[40] Transfection efficiencies were standardized by cotransfection with a reporter construct containing a β-galactosidase gene, and percent infection of cells was determined by staining with 5-bromo-4-chloro-3-indolyl-β-D-galacto pyranoside. Fold induction of the E2F or p53 reporter was calculated by scintillation counting of the samples.

Flow Cytometry

Cell lines growing in 10% FCS for at least 48 hours were prepared for flow cytometry as previously described using a 2-hour pulse of 2′,5′-bromodeoxyuridine (10 μmol/L) before harvesting.[41] Cells demonstrating less than the diploid content of DNA were excluded from the measurement of the percentages of cells in each cell cycle phase.

Statistical Analyses

The mean number of apoptotic deaths was analyzed using ANOVA for multiple comparisons. A two-tailed unpaired Student's *t* test has been used for all comparisons between two groups of cell lines. Where cells are compared within a group, eg, different treatments of plaque cells, we have used a one-way ANOVA. Where cells have been compared between groups, a two-way ANOVA has been used.

Results

Human VSMCs were derived from patients at coronary atherectomy, and normal VSMCs were obtained from recipients of heart transplantation for nonischemic cardiomyopathy. Plaque and normal VSMCs were cultured in parallel, and baseline characteristics of cell proliferation (percentage of cells in S phase and number of divisions over 24 hours) were obtained by flow cytometry and time-lapse videomicroscopy (Table 1). This confirmed that plaque VSMCs under these conditions of culture show reduced S-phase percentage and a lower number of divisions over 24 hours than did VSMCs from normal vessels. Plaque VSMCs underwent senescence, on average, at four to five passages earlier than did cells from normal vessels (Table 1), and flow cytometry showed that "senescent" cells were arrested in G_0/G_1 phase for both plaque and normal VSMCs (data not shown).

Plaque VSMCs Express Higher Levels of Hypophosphorylated RB

Cell cycle progression from G_1 to S phase is usually accompanied by phosphorylation of RB in late G_1 phase, leading to release of E2F. Conversely, hypophosphorylated RB sequesters E2F, preventing the transcription of E2F target genes. Therefore, we examined by Western blot analysis the relative amounts of hypophosphorylated and phosphorylated forms of RB in plaque and normal VSMCs in "exponential" phase culture at the same passage number (passage 3). Fig 1 demonstrates that there are approximately equal amounts of hypophosphorylated and phosphorylated RB in plaque

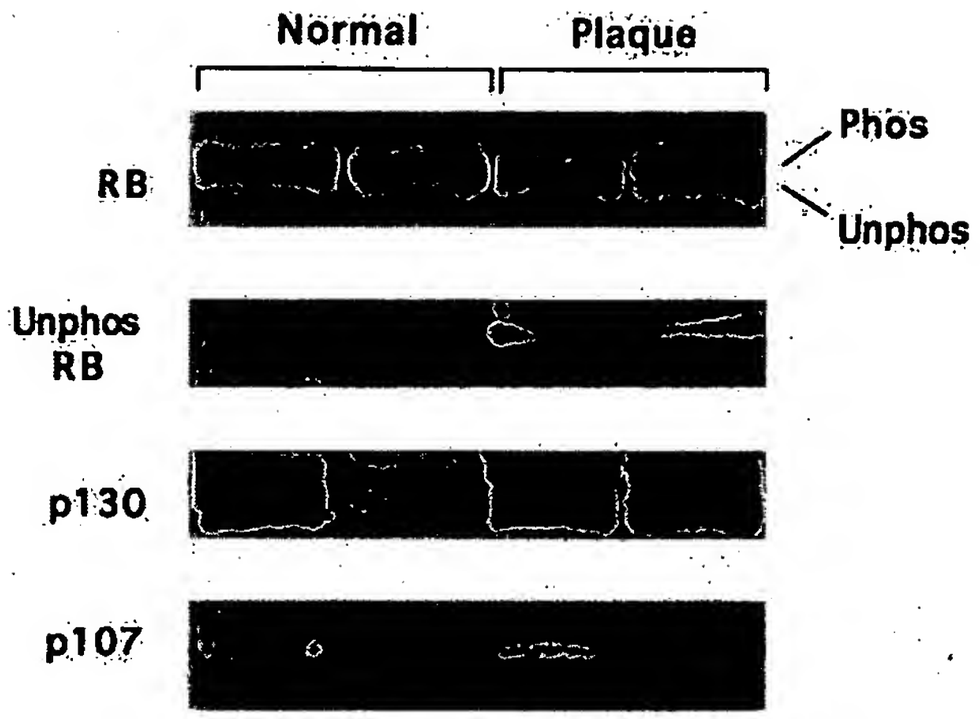

Figure 1. Top, Western blots of lysates from normal and plaque VSMCs (2 separate patients each) for RB. The upper band represents phosphorylated (Phos, inactive) RB, and the lower band represents hypophosphorylated (Unphos) RB, the form which binds E2F. Plaque VSMCs show approximately equal amounts of RB in both forms, whereas the majority of RB in the normal VSMCs is in the Phos form. Middle, Western blot (similar to the above) but using an antibody that recognizes only the Unphos form of RB, showing that significant levels of this form are only seen in plaque VSMCs. Bottom, Western blots for p130 and p107, demonstrating no difference in expression or phosphorylation of these proteins in plaque vs normal VSMCs.

VSMCs. In contrast, almost all of the RB present in normal VSMCs was present in the inactive (phosphorylated) form. Scanning densitometry of Western blots from four plaque and four normal cell lines determined that the ratio of phosphorylated to hypophosphorylated RB was ≈9:1 in normal VSMCs and 1.2:1 in plaque VSMCs (not shown). These data indicate a fundamental defect of RB phosphorylation in plaque VSMCs.

To confirm that plaque VSMCs show a higher expression of RB in the unphosphorylated form than do normal VSMCs, we analyzed RB expression on Western blot using an antibody that recognizes only the unphosphorylated form of RB. Fig 1 demonstrates that significant expression of unphosphorylated RB was seen only in plaque VSMCs. To examine whether this failure to phosphorylate RB was a generalized defect of phosphorylation of RB family members or was restricted to RB itself, we studied the expression of p107 and p130, which can both bind E2F family members. In contrast to RB, we found no difference in expression or phosphorylation status (represented by multiple bands) of p107 or p130 (Fig 1).

An increase in active (hypophosphorylated) RB in plaque VSMCs would be predicted to be associated with a decrease in E2F transcriptional activity, since E2F is normally bound to hypophosphorylated RB, thus inhibiting transactivation of E2F target genes. To examine whether the lower ratio of phosphorylated to hypophosphorylated RB in plaque VSMCs correlated with a reduction in transcriptional activation of E2F, both plaque and normal VSMCs were cotransfected with the reporter plasmid E2F-CAT, which contains multiple copies of the E2F DNA recognition site consensus sequence. Fig 2 shows that plaque VSMCs have ≈40% less E2F transcriptional activity than do normal VSMCs. Thus, when

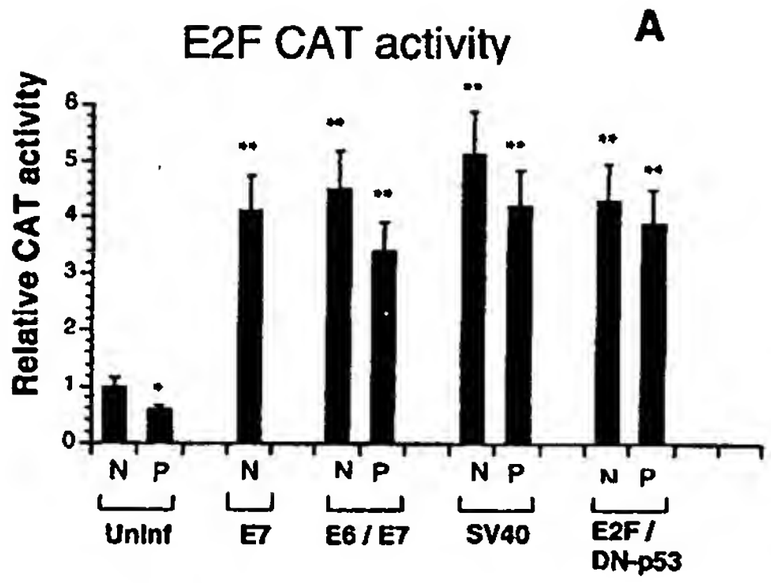

Figure 2. A, Relative E2F CAT activity in uninfected (Uninf) normal (N) and plaque (P) VSMCs and cell lines. B, Relative p53 CAT activity for uninfected (Uninf) normal (N) and plaque (P) VSMCs and derived cell lines. Uninfected VSMCs or cell lines infected with E6, E7, DN-p53, E6/E7, SV40, or E2F-1/DN-p53 were transfected with an E2F or p53 reporter construct, E2F CAT, or p53 CAT, and CAT activity was measured at 48 hours. Representative data for one uninfected plaque or normal cell line for each manipulation are shown. Values given are means of three separate uninfected or infected cell lines; error bars represent standard deviations. An arbitrary value of 1 for CAT activity is given to the first uninfected normal VSMC line. No data for plaque E7 cells are given because cultures underwent massive apoptosis and could not be maintained unless p53 was also suppressed by E6. $^{*}P<.05$ and $^{**}P<.01$ vs uninfected normal or plaque VSMCs.

compared with normal VSMCs, plaque VSMCs contain relatively higher levels of the form of RB that binds E2F and have correspondingly lower levels of E2F activity.

Generation of Human VSMCs With Low Levels of RB or p53 Activity

To examine the roles of RB and p53 in VSMCs, we generated cell lines in which RB or p53 activity was inhibited by virus gene products HPV E6, HPV E7, SV40 or by DN-p53. We also expressed ectopic E2F-1 from a retrovirus promoter to bypass RB sequestration of E2F. The effects of these manipulations on RB and p53 activity are summarized in Table 2 and are shown in Fig 2. Thus, expression of HPV E7, E6/E7, SV40, or E2F-1 increased E2F transcriptional activity in a manner consistent with inhibition of RB. In contrast, HPV E6, E6/E7, SV40, or the DN-p53 construct all inhibited p53 transcriptional activity.

Inhibition of RB Alone

To examine the effects of inhibiting RB activity alone in VSMCs, we compared the growth characteristics of normal and plaque VSMCs infected with HPV E7, which disrupts

TABLE 2. Effect of Introduced Gene Products on RB or p53 Activity

	RB	p53
HPV E6	No effect	Suppress
HPV E7	Suppress	No effect
HPV E6/E7	Suppress	Suppress
SV40	Suppress	Suppress
E2F-1	Suppress	No effect
DN-p53	No effect	Suppress

RB/E2F complexes and inactivates RB suppression of E2F. Normal VSMCs expressing E7 showed enhanced rates of proliferation, and senescence was delayed to 25 to 28 passages. In contrast, plaque VSMCs infected with E7 showed massive apoptosis (Fig 3), and cultures could not be passaged further. Cultures expressing the retrovirus vector alone showed no difference in rates of cell proliferation, apoptosis, or senescence compared with uninfected cells (not shown). Thus, inhibition of RB alone was sufficient to increase cell proliferation and significantly prolong lifespan in normal VSMCs but not in plaque VSMCs.

Inhibition of p53 Alone

To examine the effects of inhibiting p53 activity alone in VSMCs, we compared the growth characteristics of normal and plaque VSMCs expressing HPV E6, which induces degradation of p53, or the DN-p53 protein. Normal VSMCs expressing E6 but not DN-p53 showed a slightly increased rate of cell proliferation. However, the lifespan of cultures was significantly prolonged to passages 24 to 28 by both manipulations. In contrast, plaque VSMC cultures expressing either E6 or DN-p53 showed no increase in proliferation and no increase in passage number compared with uninfected cells (Table 3). Apoptosis in either normal or plaque VSMCs was not inhibited by DN-p53, although there was a small increase in apoptosis in both plaque and normal E6 cells. These data indicate a fundamental difference in the response of plaque and normal VSMCs to inhibition of p53 and also

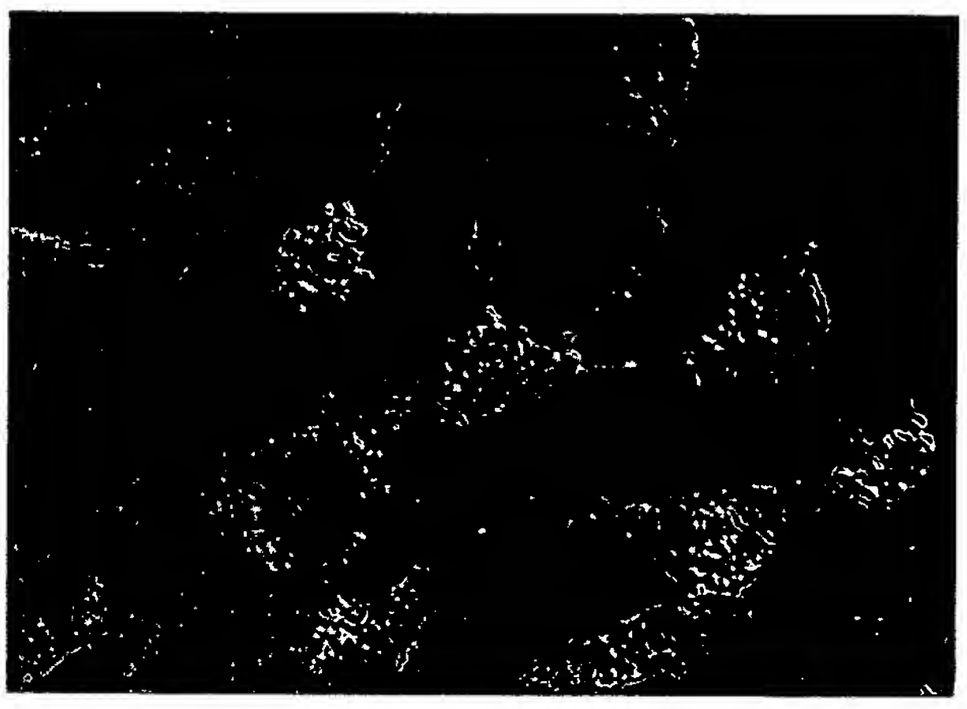

Figure 3. Time-lapse videomicroscopic appearance of human plaque E7 VSMCs undergoing apoptosis. Arrows show evidence of membrane bleb formation.

TABLE 3. Cell Division, Cell Death, S-Phase Percentage, and Passage Number at Senescence for Normal and Plaque VSMCs and Cell Lines

	No. of Divisions/24 h	No. of Deaths/24 h	S Phase, %	Passage Number at Senescence
Normal				
Uninfected	14.9±2.2	0.2±0.05	8.5±1.1	8–10
E6	23.5±3.2*	1.3±0.1*	18.2±2.1*	25–28†
DN-p53	13.2±1.8	0.3±0.08	11.8±1.8	24–26†
E7	35.7±4.1†	8.1±1.0†	22.1±3.1*	25–28†
SV40	40.1±5.8†	5.5±0.9†	26.8±3.4*	28–32†
E6/E7	38.9±4.7†	4.5±0.6†	27.2±1.8*	28–32†
E2F-1/DN-p53	37.6±5.1†	4.8±0.8†	23.2±2.4*	28–32†
Plaque				
Uninfected	4.2±1.2	3.2±1.0	3.5±1.2	3–6
E6	7.4±2.2	6.8±0.8*	8.5±1.2	3–6
DN-p53	5.4±1.7	3.8±0.9	4.1±1.1	3–6
E7	...	...	...	
SV40	18.2±1.6†	10.3±1.7*	18.2±1.9†	25–28†
E6/E7	19.1±1.9†	10.5±1.1*	21.3±2.6†	25–28†
E2F-1/DN-p53	16.5±1.8*	9.5±1.3*	20.1±2.8†	25–28†

Number of divisions over 24 hours for 100 cells by time-lapse videomicroscopy is shown for normal and plaque VSMCs and derived cell lines. S-phase percentage of plaque and normal VSMCs or cell lines was measured in cell cultures in medium containing 10% FCS. Passage number at terminal senescence was determined by extended time-lapse videomicroscopy as the point at which no detectable cell proliferation occurred after 7 days and at which no detectable S-phase percentage was present. Values are mean±SD (n=3).

*$P<.05$ and †$P<.01$ vs uninfected normal or plaque VSMCs.

demonstrate that p53 activity is not required for apoptosis of either cell type.

Inhibition of p53 and RB

To assess the effects of inhibiting both RB and p53 activity, we compared the growth characteristics of normal and plaque VSMCs infected with retroviruses encoding SV40 or E6/E7. These manipulations were shown to increase E2F transcriptional activity (as a marker of inhibition of RB) and also inhibited p53 activity (Fig 2). Normal VSMCs expressing SV40 or E6/E7 showed increased rates of proliferation, and cells could be maintained to passages 28 to 32. At this point, cultures expressing both SV40 or E6/E7 underwent crisis, characterized by no cell proliferation for ≈2 weeks and massive apoptosis. However, surviving cells began to proliferate thereafter, and cells could be maintained indefinitely (to >60 passages to date). Plaque VSMCs expressing SV40 or E6/E7 also showed increased rates of cell proliferation, and cells could be maintained to passages 25 to 28. At this point, crisis occurred, and plaque cultures could not be immortalized (Table 3).

Expression of E2F-1 and Suppression of p53

SV40 and E6/E7 are promiscuous gene products, which bind to a range of intracellular targets, including other members of the RB family of pocket proteins, p107 and p130. To determine whether the effects of SV40 and E6/E7 could be reproduced by bypassing RB repression of E2F, coupled with abrogation of p53 function, we overexpressed E2F-1 in plaque and normal VSMCs in which we had already suppressed p53 transcriptional activity by DN-p53. Overexpression of E2F-1 in combination with DN-p53 resulted in plaque and normal VSMCs that underwent increased cell proliferation and could be maintained to passages 25 to 28 and 28 to 32, respectively. In contrast, expression of E2F-1 alone in plaque VSMCs resulted in massive apoptosis of cells, and immortalized cells could not be produced (not shown), similar to the effects of E7. These data confirm our previous finding that plaque VSMCs have a markedly increased sensitivity to p53-mediated apoptosis.[35] Interestingly, the combination of E2F-1 and DN-p53 could not fully immortalize either normal or plaque VSMCs.

Discussion

Compared with normal VSMCs, VSMCs derived from atheromatous plaques show three characteristic properties in culture: (1) lower rates of proliferation, (2) senescence at earlier passage numbers, and (3) increased rates of spontaneous apoptosis. In other cell types, these processes are regulated by RB and p53, such that inhibition of RB can result in increased cell proliferation and a p53-dependent apoptosis. We have previously demonstrated that suppression of p53 activity alone in plaque VSMCs does not increase cell proliferation but that plaque VSMCs are very sensitive to p53-mediated apoptosis.[35] The present study was therefore designed to determine to what extent differences in p53 and RB activities account for differing growth characteristics of the two cell types. We find that p53 and RB in combination

are involved in the control of cell proliferation and cell senescence in human VSMCs, with RB exerting major effects in controlling cell proliferation and cell senescence and p53 affecting apoptosis.

Role of RB

We find that plaque VSMCs, which undergo slower proliferation and early senescence, have a lower ratio of phosphorylated to hypophosphorylated RB than do normal VSMCs. Hypophosphorylated RB is the form that sequesters E2F and suppresses cell proliferation. Indeed, plaque VSMCs have lower levels of E2F transcriptional activity than do normal VSMCs. We also show that RB is directly responsible for inhibiting proliferation and inducing senescence in plaque VSMCs and that the relative increase in hypophosphorylated RB in plaque VSMCs is not just a consequence of senescence. Thus, inhibition of RB binding to E2F by E7 promotes cell proliferation and delays senescence in plaque VSMCs. Although it is possible that the action of E7 occurs partly independent of RB, a similar increase in cell proliferation and delay of senescence occurs in plaque VSMCs by ectopic expression of E2F-1. However, when plaque VSMCs have normal p53 activity, the proproliferative effect of inhibiting RB (by E7) is masked by a massive p53-mediated apoptosis, and plaque VSMCs expressing ectopic E2F-1 alone also undergo apoptosis. However, when p53 is also inhibited in plaque E7 or E2F-1 cells (by E6 or DN-p53, respectively), inhibition of RB can be seen to promote both cell proliferation and delay senescence.

We also find that RB inhibits proliferation and induces senescence in normal VSMCs, since suppression of RB by E7 in normal VSMCs increases cell proliferation and delays senescence. Indeed, in contrast to plaque VSMCs, inhibition of RB is all that is required to significantly increase the lifespan of cultures. Although expression of E7 or ectopic expression of E2F-1 does increase apoptosis in normal VSMCs, the relative insensitivity of normal VSMCs to p53-mediated apoptosis means that the proproliferative action of E7 or E2F-1 predominates.

It is not apparent from our experiments why plaque VSMCs should show a defect in phosphorylation of RB. RB is normally phosphorylated in G_1 phase by the action of cdk(2/4)/cyclin (E/D) complexes. Activation of both cyclin D_1–and cyclin E–associated kinases occurs in response to early gene activation, such as mitogen-stimulated induction of c-*myc*.[42] Cdk activity can also be suppressed by interactions between cdk inhibitors (p16, p21, and p27) and by phosphorylation at specific threonine and tyrosine residues. Conversely, dephosphorylation of these residues activates cdks. This dephosphorylation is mediated by cdc25 phosphatases, and the physiological substrates of cdc25A have been identified as cdk2 and possibly cdk4, implicating cdc25A as a likely regulator of cdk phosphorylation of RB. Thus, the defect in RB phosphorylation that we see in plaque VSMCs could occur at the level of activation of many possible proteins. However, phosphorylation of RB is a critical step in regulating plaque VSMC proliferation and senescence, as seen by the finding that disruption of RB/E2F complexes in plaque VSMCs promotes cell proliferation and delays senescence, rendering a plaque VSMC more like a normal VSMC, as long as apoptosis can be inhibited.

Role of p53

In contrast to RB, no difference in p53 activity was observed between normal and plaque VSMCs, and inhibition of endogenous p53 alone (by DN-p53) has minimal effects on cell proliferation or apoptosis in either plaque or normal VSMCs, although E6 expression may increase apoptosis slightly. These findings indicate that spontaneous apoptosis of human VSMCs in culture is not regulated by endogenous levels of p53. However, we find that p53 is a potent inducer of apoptosis in VSMCs that have deregulated expression of E2F-1 or when RB is inhibited by E7. Since normal human VSMCs are less sensitive to p53-mediated apoptosis,[35] cultures of normal VSMCs with low levels of RB activity are still able to proliferate and expand. In contrast to RB, inhibition of p53 alone in plaque VSMCs does not delay senescence, although it does prolong the lifespan in normal VSMCs. This further emphasizes that cell proliferation and cell senescence in plaque VSMCs are related to the defect in RB phosphorylation seen in plaque VSMCs rather than any difference in p53 activity.

Regulation of VSMC Senescence

Normal human diploid cells undergo senescence in culture after a variable number of divisions. A characteristic feature of senescent human diploid fibroblasts is the inability to phosphorylate RB.[43,44] Our studies indicate that an identical defect is responsible for early plaque VSMC senescence. We also provide evidence that full immortalization of plaque VSMCs, with generation of a cell line, may require expression of additional gene products. Normal VSMCs expressing SV40 or E6/E7 could be maintained through a "crisis" period, characterized by reduced proliferation and massive apoptosis, until the spontaneous development of immortal cells. In contrast, plaque VSMCs could not be maintained through crisis despite continued expression of E6/E7 or SV40. Although most of the activity of E6/E7 and SV40 could be reproduced by the expression of E2F-1 and suppression of p53 (DN-p53), permanent cell lines of either normal or plaque VSMCs could not be obtained with E2F-1/DN-p53. This indicates that there may be additional activity conferred by E6/E7 and SV40 that allows transformation to an immortalized phenotype. Although the requirements for immortalization of VSMCs have dubious biological significance, our data indicate that suppression of RB and p53 is insufficient to generate an immortalized plaque VSMC line.

Both RB and p53 activity have previously been implicated in cellular senescence. Thus, ectopically expressed RB induces growth arrest and senescence in various cell types (eg, see Reference 45), whereas ectopic expression of E2F-1 can overcome senescence.[46,47] Interestingly, although E2F-1 appears to overcome RB-mediated senescence, by blocking RB repression of promoters containing E2F sites, E2F-1 inhibits RB-mediated growth G_0/G_1 arrest by direct transactivation of E2F target genes.[47] Some p53-deficient cells also escape readily from senescence

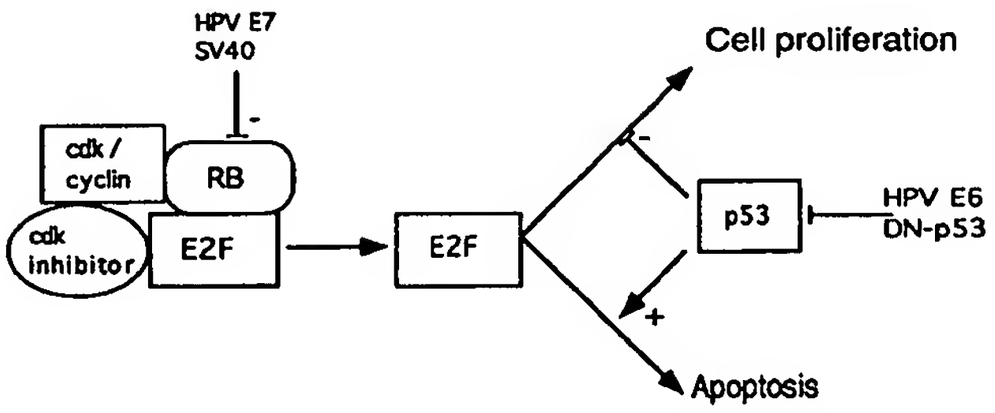

Figure 4. Model of RB/p53 interactions to regulate cell cycle transit and apoptosis in human VSMCs. Entry from G_1-S phase is mediated in large part by RB interactions with the E2F transcription factor family. Growth factors lead to the phosphorylation of RB in late G_1 phase by cdk/cyclin. Phosphorylation of RB releases E2F, allowing transcriptional activation of E2F target genes, which promotes S-phase entry and cell proliferation. HPV E7 and SV40 promote release of E2F from RB, whereas HPV E6 and DN-p53 inhibit p53 activity. In normal VSMCs, release of E2F is all that is required to promote cell proliferation. In plaque VSMCs, E2F release also induces apoptosis if p53 is active. Successful completion of the cell cycle in human plaque VSMCs thus requires suppression of p53 proapoptotic activity in addition to phosphorylation of RB.

and undergo immortalization. Thus, transdominantly acting mutant forms of p53 can extend lifespan, although further oncogene disruptions are often needed for immortalization to occur.[13,48]

The cooperative interactions of RB and p53 in regulating senescence and apoptosis have been demonstrated in other cell types. In human fibroblasts, prolongation of culture lifespan occurs when RB is inhibited with antisense oligonucleotides but not with oligonucleotides to p53; however, the inhibition of p53 promoted RB effects.[49] In addition, apoptosis induced by p53 can be overcome by suppression of RB,[29,50] and apoptosis due to RB deficiency can be blocked by inhibiting p53.[50–54] Furthermore, the growth-suppressive effect of p53 is mediated by active RB.[55] These results indicate that the loss of RB and p53 activity together is a particularly potent combination to increase cell number, resulting in both an increase in cell proliferation and an inhibition of apoptosis. Conversely, the increased RB activity found here and the increased sensitivity to p53-mediated apoptosis of plaque VSMCs found in earlier studies[35] are a particularly potent combination to inhibit an increase in cell number in atherosclerotic plaques.

These observations allow us to present a model of the control of cell cycle transition and apoptosis in human VSMCs by the cooperative interactions between RB and p53 (Fig 4). In normal VSMCs, phosphorylation of RB in late G_1 phase by cyclin-dependent kinases releases E2F from RB. Free E2F can then transcriptionally activate its target genes, many of which are required for entry into S phase. To increase cell proliferation, normal VSMCs require only disruption of RB/E2F complexes. p53 inactivation in normal VSMCs does prolong lifespan but has little effect on cell proliferation. In contrast, inactivation of RB in plaque VSMCs induces massive apoptosis, which is mediated by p53. Suppression of p53 activity alone does not increase proliferation of plaque VSMCs, but suppression of p53 blocks apoptosis. Thus, the combination of inhibition of RB and inhibition of p53 is required to increase cell proliferation of plaque-derived VSMCs.

Our observations have important implications for the control of VSMC number in the vessel wall in disease states such as atherosclerosis and restenosis after angioplasty. Thus, it is likely that any agent that is capable of inducing phosphorylation of RB with release of E2F will successfully induce cell proliferation in normal VSMCs. Growth factors, such as platelet-derived growth factor, epidermal growth factor, and fibroblast growth factor, have been shown to induce RB phosphorylation in quiescent cells on entry into the cell cycle (reviewed in Reference 56). In contrast, the same mitogenic stimulus resulting in E2F release in plaque VSMCs is also likely to induce apoptosis. To complete the cell cycle, plaque VSMCs require suppression of p53 activity in addition to phosphorylation of RB. This may explain why growth factors such as PDGF found in plaques do not result in higher levels of cell proliferation in VSMCs than are seen in normal vessels.[1] In contrast, an increased rate of apoptosis in VSMCs is found in plaques compared with normal vessels.[6,7]

The role of cell senescence in vessel wall biology is difficult to predict. In the present study, we have sampled VSMCs from advanced plaques only. We find that these VSMCs undergo senescence very readily in culture, in some cases within a few days of isolation. Although this does not indicate that VSMCs do not proliferate in the pathogenesis of atherosclerosis, it does suggest that VSMCs may have a low capacity for cell division in advanced plaques in vivo. The fibrous cap region of the atherosclerotic plaque is composed largely of VSMCs, and the structural integrity of the cap determines whether the plaque is likely to undergo rupture. Clearly, if the cap sustains repeated damage, as is likely in complex atherosclerotic lesions, and the resident VSMCs are both unable to replicate and undergo apoptosis, then further weakening of the plaque will ensue.

In conclusion, we have demonstrated that VSMC proliferation, senescence, and apoptosis are controlled, at least in part, by RB and p53 expression. The lower rate of cell proliferation and early senescence of plaque VSMCs are due to decreased phosphorylation of RB, with consequent reduced E2F-driven S-phase entry. Since plaque VSMCs show an increased sensitivity to p53-mediated apoptosis, successful proliferation and an increase in lifespan of plaque VSMCs require abrogation of RB to enhance cell proliferation and abrogation of p53 to block apoptosis. Thus, suppression of the activity of both RB and p53 may be required for human VSMCs in plaques to undergo successful cell proliferation. Our data also emphasize the concept that an increase in VSMC number in the vessel wall may require the coordinated action of agents that both increase cell proliferation and suppress apoptosis.

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